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Folate bioavailability

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The achievement of optimal folate status to prevent neural-tube defects, and possibly other diseases, is hindered by the well-recognised incomplete bioavailability of the natural folates found in foods compared with the synthetic vitamin, folic acid. Folate bioavailability from different foods is considered to be dependent on a number of factors, including the food matrix, the intestinal deconjugation of polyglutamyl folates, the instability of certain labile folates during digestion and the presence of certain dietary constituents that may enhance folate stability during digestion. There is conflicting evidence as to whether the extent of conjugation of polyglutamyl folate (in the absence of specific inhibitors of deconjugation in certain foods) is a limiting factor in folate bioavailability. Estimates of the extent of lower bioavailability of food folates compared with folic acid (relative bioavailability) show great variation, ranging anywhere between 10 and 98%, depending on the methodological approach used. The lack of accurate data on folate bioavailability from natural food sources is of particular concern in those countries in which there is no mandatory folic acid fortification, and therefore a greater reliance on natural food folates as a means to optimise status. Apart from the incomplete bioavailability of food folates, the poor stability of folates in foods (particularly green vegetables) under typical conditions of cooking can substantially reduce the amount of vitamin ingested and thereby be an additional factor limiting the ability of food folates to enhance folate status. A recent workshop convened by the Food Standards Agency concluded that gaining a better understanding of folate bioavailability in representative human diets is a high priority for future research.

Food folate: Folic acid: Folate bioavailability methodology: Folate requirements

Folate bioavailability refers to the proportion of ingested folate that is absorbed and becomes available for metabolic processes or storage. This B-vitamin has been attracting major scientific and public health interest in recent years. Apart from its well-established role in the prevention of neural-tube defects (Medical Research Council Vitamin Study Research Group, 1991; Czeizel & Dudas, 1992), newer potential roles for folate in protecting against CVD (Wald *et al.* 2002; Strain *et al.* 2004), certain cancers (Choi & Mason, 2000) and neuro-psychiatric conditions (Seshadri *et al.* 2002) are emerging. However, when dietary intakes are viewed from the perspective of optimising folate status to levels associated with lowest risk of disease, rather than merely preventing overt folate deficiency (i.e. megaloblastic anaemia), it is clear that typical folate intakes are suboptimal in the diets of many

individuals. This widespread under-provision of folate is generally attributed to the poor bioavailability of natural food folates. Although there is general agreement among experts that the bioavailability of natural food folates is incomplete when compared with the synthetic vitamin, folic acid (FA), the extent to which bioavailability is reduced is somewhat controversial. Estimates of the extent of lower bioavailability of food folates compared with FA (relative bioavailability) show great variation in human studies, ranging anywhere between 10 and 98% (Tamura & Stokstad, 1973; Coleman *et al.* 1975; Baker *et al.* 1978; Sauberlich *et al.* 1987; Brouwer *et al.* 1999; Prinz-Langenohl *et al.* 1999).

As a result of the major current focus and debate on FA fortification of foods, the issue of incomplete bioavailability of natural folates may be considered unimportant.

Abbreviations: DFE, dietary folate equivalents; FA, folic acid; GCPH, glutamate carboxypeptidase II.

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However, it remains a fundamental concern for two reasons. First, there are many countries that do not have legislation in place for the mandatory fortification of grain food with FA similar to that introduced in recent years in North America (Fletcher *et al.* 2004). Such countries are therefore reliant mainly on natural food folates as a means to optimise status. Second, irrespective of whether a country has a FA fortification policy, the issue of setting dietary folate recommendations remains an ongoing concern for policy makers. The focus here has generally shifted in recent years from the traditional approach of preventing overt deficiency to the newer evidence-based approach of setting dietary requirements on the basis of optimal health. However, in the case of folate, recommendations based on an adjustment for the differences in bioavailability between natural food folates and the synthetic vitamin are emerging, with the recent introduction of 'dietary folate equivalents' (DFE; Institute of Medicine, 1998) in the USA. The DFE is defined as the quantity of natural food folate plus 1.7 times the quantity of FA in the diet; this definition is based on the assumption that the bioavailability of FA added to food is greater than that of natural food folate by a factor of 1.7. This estimation is, however, highly dependent on one metabolic study in non-pregnant women that estimated the bioavailability of food folates to be $\leq 50\%$ relative to that of FA (Saubert *et al.* 1987), and other evidence showing that FA added to food has about 85% of the bioavailability of free FA (Pfeiffer *et al.* 1997). Other countries wishing to consider the use of this approach or a similar one for setting dietary folate recommendations require accurate and meaningful folate bioavailability data in order to confirm the robustness of the US DFE value.

The present review will address the various factors that are considered to contribute to the incomplete bioavailability of natural folates from foods, the experimental approaches that have been used to examine food folate bioavailability, the major findings from recent bioavailability studies in human subjects and future research priorities.

Factors considered to contribute to the incomplete bioavailability of natural food folates

The bioavailability of folates from various foods is considered to be dependent on a number of factors, including: intestinal deconjugation of polyglutamyl folates; the food matrix; the instability of certain labile folates during digestion (or before ingestion); the presence of certain dietary constituents that may enhance folate stability during digestion (e.g. folate-binding proteins, ascorbate).

Factors related to intestinal deconjugation of food folates

In contrast to synthetic FA, which is a monoglutamate, natural food folates are predominantly polyglutamates with a variable number of glutamate residues. A recent study from The Netherlands has estimated that approximately two-thirds of total folate intake from a mixed unfortified diet is in the polyglutamyl form, derived mainly from

vegetables (Melse-Boonstra *et al.* 2002). These polyglutamates need to be hydrolysed to the monoglutamate form for normal absorption in the proximal small intestine. This process is controlled by the intestinal brush-border enzyme glutamate carboxypeptidase II (GCPII).

GCPII has a pH optimum at 6–7 (Halsted, 1990) and ingestion of foods that can change the intestinal pH may result in incomplete intestinal deconjugation of polyglutamyl folates. For example, Tamura *et al.* (1976) have found a marked reduction in the bioavailability of heptaglutamate when added to 600 ml diluted orange juice in comparison with FA. The authors have established that the reduction in heptaglutamate bioavailability is as a result of the lowering of pH (to 3.7) by the high load of orange juice. They have shown that heptaglutamate bioavailability is reduced when citric acid is added to orange juice, but when the same mixture is neutralised to pH 6.4 the bioavailability of heptaglutamate does not differ from that of FA (Tamura *et al.* 1976). In addition, some food components and certain drugs have been reported to act as inhibitors of the activity of GCPII. Studies with stable isotopes in human subjects (Wei *et al.* 1996) and *in vitro* experiments with porcine jejunal brush-border membrane (Wei & Gregory, 1998) indicate that organic acid ions (citrate, malate, ascorbate and phytate) present in orange juice have a combined inhibitory effect on the activity of GCPII. Nucleic acids in yeast ingested in large doses have also been reported to decrease the activity of GCPII (Rosenberg & Godwin, 1971). A naturally-occurring heat-activated GCPII inhibitor has been identified in the skin of various pulses (Butterworth *et al.* 1974); however, later bioavailability studies with human subjects have failed to detect any effect of beans on the utilisation of heptaglutamates (Keagy *et al.* 1988). Some drugs (salicylazosulphapyridine) and ethanol have also been reported to competitively inhibit GCPII (Halsted, 1990).

Recently, a polymorphism (C1561T) in the gene encoding GCPII has been identified and *in vitro* studies have found that COS-7 cells expressing the mutant variant have a 53% lower GCPII enzyme activity than cells with the wild genotype (Devlin *et al.* 2000). However, the frequency of the homozygous mutant genotype for this polymorphism in the general population is very low (0.5%), and its *in vivo* effects on dietary folate digestion and folate status remain unclear, since various human studies have shown contradictory results (Devlin *et al.* 2000; Lievers *et al.* 2002; Vargas-Martinez *et al.* 2002; Afman *et al.* 2003).

The question as to whether polyglutamyl folates are inherently less bioavailable than monoglutamyl folates (i.e. in the absence of specific inhibitors of deconjugation such as organic acids) remains to be resolved. The bioavailability of folate polyglutamates relative to monoglutamates has been explored in different studies with various results. Earlier acute studies (Godwin & Rosenberg, 1975; Keagy *et al.* 1988), together with a recent chronic study by Melse-Boonstra *et al.* (2004), have indicated that the bioavailability of polyglutamyl folate is markedly lower than that of monoglutamyl folate. In contrast, recent results (Hannon-Fletcher *et al.* 2004) have found no evidence to support the view that the extent of glutamation *per se* is a

limiting factor in the bioavailability of folates from natural food sources. A 6-week intervention study with healthy subjects has shown no difference in the bioavailability of folates from yeast (providing 100% of the folate as polyglutamyl folate) and spinach (*Spinacia oleracea*; providing 50% of the folate as polyglutamyl folate). These observations are in good agreement with previous findings (Wei *et al.* 1996) from studies using exogenous ^2H -labelled monoglutamyl and polyglutamyl folates added to various foods that have shown equivalent bioavailability for the two forms of folate. Similar findings have also been reported by Konings *et al.* (2002) in an acute study of subjects with an ileostomy that compared folate absorption from spinach containing 60% of the folate as polyglutamate and spinach pretreated before ingestion to convert the folates to the monoglutamate form. All such findings showing a similar bioavailability for polyglutamyl and monoglutamyl folate forms are consistent with earlier observations (Halsted, 1990) indicating that the activity of the human jejunal brush-border enzyme GCPH exceeds that needed for hydrolysis of polyglutamyl folates within the range of dietary intake and is, therefore, not rate limiting in the absorption process.

Factors related to the food matrix

Since folates are covalently bound to macromolecules in foods, the food matrix as a whole and its components can also influence folate bioavailability by entrapment in the matrix, thereby hindering diffusion to the absorptive surface during digestion. Incomplete release from plant cellular structure may be a factor affecting folate bioavailability in certain plants. Two independent studies have demonstrated that folate bioavailability from minced, chopped or enzymically-liquefied spinach is higher than that from whole spinach leaves when equal amounts of spinach folates are provided to human volunteers (van het Hof *et al.* 1999; Castenmiller *et al.* 2000). The effects of wheat bran and dietary fibre on folate bioavailability have been explored in various acute and longer-term studies in human subjects, but the results are inconsistent (Bailey *et al.* 1988; Keagy *et al.* 1988; Castenmiller *et al.* 2000). *In vitro* studies with intestinal brush-border-membrane vesicles have indicated that dietary fibre does not affect the activity of GCPH (Bhandari & Gregory, 1990).

Factors related to chemical instability of food folates

Unlike FA, which is a fully-oxidised molecule, natural folates are a mixture of reduced forms with or without C_1 substitution of the pteridine ring. The chemical structure of FA renders it very stable to oxidation and resistant to destruction (Colman *et al.* 1975), whereas natural folates are labile and prone to oxidative cleavage at the C-9–N-10 bond (Murphy *et al.* 1976). There are two consequences in relation to folate availability. First, extensive losses of folate can occur during cooking and preparation of foods, which may considerably reduce the amount of folate ingested. Folate losses in the range of 50–80% have been reported in green vegetables after boiling (Hurdle *et al.* 1968; Leichter *et al.* 1978; De Souza & Eitenmiller, 1986;

McKillop *et al.* 2002) and in processed legumes (Dang *et al.* 2000). In addition to any folate losses before ingestion, considerable instability of certain labile food folates has been demonstrated in studies *in vitro* that mimic the intestinal digestion of folates from various foods (Seyom & Selhub, 1998). Thus, the instability of labile folate species *in vivo* may be an important factor contributing to the incomplete bioavailability of natural food folates.

As indicated by previous studies (Smith *et al.* 1985; Andersson & Oste, 1992), some dietary constituents may enhance folate bioavailability by increasing the stability of food folates during food processing and during digestion in the gastrointestinal tract (e.g. folate-binding protein and antioxidants such as ascorbic acid).

Other potential factors affecting food folate bioavailability

Apart from potential factors affecting intestinal deconjugation, food matrix effects or chemical instability of food folates, the presence of other food components in the intestinal milieu may inhibit folate absorption. For example, Pfeiffer *et al.* (1997) have reported a small reduction in the absorption of [^{13}C -5]FA when it is administered after a light breakfast meal compared with its administration without food.

Experimental approaches to determining food folate bioavailability

The bioavailability of natural folates has been studied using various acute and chronic protocols that involve the administration of reduced folates either in the form of foods or commercial preparations corresponding to the major food folate forms (usually 5-methyltetrahydro-folate). Typically the bioavailability of reduced folates is compared with that of FA, since the latter, which is the form used for supplementation and food fortification purposes, is considered to be highly bioavailable.

Acute studies

The protocol used in acute folate bioavailability studies is similar to that employed in pharmacology for testing the pharmacokinetics of different drugs. Typically, in a cross-over or parallel design, a single ingestion of a reference dose of reduced folates or FA is administered to human volunteers and the changes in blood and/or urinary folate concentrations are monitored at various time points. The typical variables used to compare responses between the different treatments include the maximal plasma or serum folate concentration, the time required to achieve maximal plasma or serum folate response, the calculated area under the curve for plasma or serum folate response and urinary folate measurements. On the basis of the type of folate compounds administered, acute studies may be subdivided into two categories, non-isotopic and isotopic studies.

Non-isotopic acute studies. The important methodological considerations of non-isotopic acute studies have been discussed comprehensively by other investigators (Pietrzik *et al.* 1990; Gregory, 1995, 2001). These

considerations include: the administration of a folate dose sufficiently high to elicit measurable folate responses in blood or urine, but not so high that it exceeds the physiologically-relevant range; the selection of subjects with high folate status or the use of a folate presaturation protocol (otherwise the ingested dose may be preferentially directed towards depleted folate pools); frequent and substantial length (usually at least 8 h) of monitoring of the folate response after ingestion of the test dose in order to ensure that the peak response is not missed and to allow sufficient time for folate concentrations to return to baseline values; the recruitment of a sufficient number of subjects for statistical power, given the variability in the folate response; the avoidance of fasting, which is reported to provoke an increase in plasma folate levels, probably by interrupting the enterohepatic folate circulation; strict control of dietary folate intake during the treatment period; the inclusion of a placebo group to control for any changes in folate measurements as a result of the conditions of the protocol.

The earliest non-isotopic acute studies using folate compounds in the physiological range of intake reported a variety of results. Perry & Chanarin (1970) and Brown *et al.* (1973) demonstrated higher serum responses to reduced folates than to FA, whereas Tamura & Stokstad (1973) were unable to show any marked differences between natural folate forms and FA, based on 24 h urinary folate excretion. However, the validity of these early results is questionable, given the lack of placebo treatment in any of the studies and, particularly, the short period of monitoring (2–3 h), which was insufficient to quantify the full plasma folate response in the studies of Perry & Chanarin (1970) and Brown *et al.* (1973). A later acute study, using a very extended period of blood folate monitoring (3 d) and a higher folate dose (1000 µg), has reported similar bioavailabilities for 5-formyltetrahydrofolate and FA, based on the area under the curve for plasma folate response (Pietrzik & Remer, 1989). Such findings are in agreement with a recently published study (Pentieva *et al.* 2004) showing equivalent bioavailabilities for 5-methyltetrahydrofolate and FA, based on plasma folate response measured for a 10 h period and using a much lower folate dose of 500 µg.

Few studies have explored folate bioavailability from different foods in an acute model. Tamura & Stokstad (1973) have reported that folate bioavailability, measured by urinary folate excretion up to 24 h after the food consumption, varies considerably between different foods. Twelve food items were studied, of which banana and lima beans (*Phaseolus limensis*) were found to contain highly-bioavailable folates (82–96% relative to FA), whereas folates from other foods such as romaine lettuce, orange juice, cabbage, soybean meal and wheat germ have a low bioavailability (25–47%). More-recent studies, based on the determination of serum folate area under the curve, have reported that the bioavailability of folates from wheat-aleurone flour (Fenech *et al.* 1999) and from spinach (Konings *et al.* 2002) is similar to that of an equivalent amount FA provided as a tablet. The reported differences between studies in the bioavailability of folates from various foods are probably the result of differences in the

protocols used, together with any inherent differences between the foods administered, including the food matrices and other potential factors affecting folate bioavailability (GCP II inhibitors, pH).

Isotopic acute studies. Isotopic studies offer the advantage of high specificity, since the only source of the labelled folate detected in the blood and/or urine can be the ingested folate isotope (Gregory, 2001). These studies became particularly attractive after the introduction of stable-isotope folate forms considered safe for human use. However, the measurement of the labelled folates and their metabolic products requires expensive and sophisticated equipment such as GC–MS or liquid chromatography–MS. In addition, most of the stable-isotope folates are not commercially available and need to be synthesised and purified by the investigators (Gregory, 1990; Maunder *et al.* 1999). Other investigators (Gregory & Toth, 1990; Gregory & Quinlivan, 2002) have reviewed the methodological requirements for stable-isotope folate studies. Two types of protocols have been used: (1) single-tracer protocols involving the monitoring of plasma folate responses after the administration of an oral dose of labelled reduced folates or FA (Wright *et al.* 2003); (2) dual-tracer protocols in which urinary isotope excretion ratios are compared after an oral dose of labelled reduced folates or FA and an intravenous dose of differently-labelled FA (Gregory *et al.* 1992). In general, studies using both types of protocols have tended to suggest differential bioavailability of FA compared with reduced folate derivatives. However, recent isotopic studies by Wright *et al.* (2003) and Sanderson *et al.* (2003) have raised questions about the interpretation of results from acute bioavailability protocols that rely on measuring responses to the oral administration of various folates relative to a reference dose of FA. These authors have examined the rate and extent of appearance of labelled and unlabelled folates in plasma following oral doses of FA, 5-formyltetrahydrofolate or intrinsically-labelled spinach folates. The results show a marked difference in the absorption of FA *v.* the natural folates; however, all forms cause an increase in unlabelled plasma folate, and the extent of this phenomenon differs between FA and the natural folates. The authors concluded that there is a different hepatic first-pass effect between FA and the reduced folate forms, and highlighted a potential limitation with this approach arising from the displacement of unlabelled tissue folate (not originating from the oral test doses) into the plasma. Other potential limitations of this (Wright *et al.* 2003) and similar studies that may constrain their interpretation include the administration of the folate treatments at different doses, with subsequent mathematical adjustment of the response to an equivalent dose, and the fasting of subjects for >16 h, which may provoke an increase in unlabelled plasma folate by diminishing bile secretion into the gut, reported to be a major elimination route for folates (Pietrzik *et al.* 1990).

Chronic studies

Chronic bioavailability studies explore, usually in parallel design, the effect of long-term (typically 4–24 weeks)

interventions with comparable doses of FA and reduced folates (specific food folates, mixed diets or commercial preparations corresponding to the major food folate forms) on markers of folate status (serum folate, erythrocyte folate, plasma homocysteine). To avoid the effect of confounding factors on the response measurement, the following categories of subjects should be excluded from participation: users of B-vitamin supplements; users of drugs affecting B-vitamin metabolism; those suffering from chronic disease. The inclusion of a placebo group is essential to control for any changes in folate measurements as a result of the conditions of the intervention. In order to accurately compare the effects of different treatments, it is important that subjects are stratified at baseline to the various treatment groups on the basis of an end point of interest, particularly if (as in the case of plasma homocysteine) the response is likely to be influenced by baseline values. In addition, subject compliance to the intervention needs to be rigorously supervised by frequent contacts and recorded on an ongoing basis during the intervention period. Furthermore, the total folate content in the treatments being administered needs to be verified at the start and during the intervention. These aspects are often overlooked in the reporting of chronic folate bioavailability studies, but are of major relevance in the interpretation of the results. If these measures are not in place to confirm that the targetted folate levels are being delivered over the intervention period, any comparison of responses to different folate treatments or different intervention doses will be very problematic.

Studies examining the effect of chronic supplementation with commercial folate preparations at doses in the range of 100–400 µg/d have reported similar bioavailabilities for FA and 5-methyltetrahydrofolate (Venn *et al.* 2002, 2003; Lamers *et al.* 2004). However, the results of chronic studies using food folates to estimate the bioavailability of natural folates relative to FA show great variation depending on the food source and the response index used. The interpretation of bioavailability studies involving the provision of folate-rich foods to free-living subjects may be particularly problematic as a result of a number of confounding effects. These effects include the poor stability of food folates during cooking (McKillop *et al.* 2002), which results in considerable and very variable folate losses before ingestion. This factor, together with poor compliance of subjects with demanding intervention protocols in long-term studies or dietary displacement of usual food folate sources with intervention foods (Cuskelly *et al.* 1996; Ashfield-Watt *et al.* 2003), may result in considerably less food folate being consumed than that targetted. In contrast, FA treatment, against which responses to food folates are typically compared, not only provides the vitamin in a very stable form, but it is likely to be associated with much greater compliance of subjects because they are required to simply ingest a tablet daily in addition to their usual diet (Cuskelly *et al.* 1996). Such differences can exert major confounding effects that compromise the interpretation of many folate bioavailability studies in human subjects.

One landmark study, which measured non-pregnant women for 92 d in a metabolic unit, estimated the

bioavailability of natural folates from a mixed diet to be $\leq 50\%$ that of FA (Sauberlich *et al.* 1987). Although this study was very precise, the results are based on a small number of subjects (ten subjects divided into three parallel groups) and have never been confirmed in a larger-scale metabolic study. However, in the absence of a more reliable estimate, the results of the Sauberlich *et al.* (1987) study have been used as the basis to set current US dietary folate recommendations, which are now expressed as DFE values (Institute of Medicine, 1998). The concept of DFE recognises for the first time the differences in bioavailability between natural food folates and the synthetic vitamin FA that is added to foods during fortification. Recent results (Hannon-Fletcher *et al.* 2004), based on the administration of representative food folates in a controlled (but not metabolic) 6-week intervention study, have estimated the bioavailability of food folate relative to FA to be approximately 45%, consistent with the estimate of '< 50%' (Sauberlich *et al.* 1987) used as the cornerstone of the US DFE value. Also generally consistent with the US approach, chronic studies in free-living subjects that have compared the response of food folates with that of FA-fortified foods at equivalent intakes have shown the latter to be much more effective in increasing serum and erythrocyte folate (Cuskelly *et al.* 1996; Riddell *et al.* 2000) and reducing homocysteine (Riddell *et al.* 2000).

Recent folate bioavailability studies and future research priorities

The Food Standards Agency recently convened a workshop to review current research and to consider future research priorities in folate bioavailability. The full report, which includes the results of a number of recently-completed studies (many of which were Food Standards Agency funded), has been published elsewhere (Sanderson *et al.* 2003). The workshop concluded that the assessment of folate bioavailability in whole diets is a high priority for future research.

The biggest challenge in any future studies aiming to assess folate bioavailability in this way will be the delivery of food folate and FA at equivalent doses throughout the intervention period. One way in which the whole diet could be controlled would be to conduct the study in a metabolic unit. However, this approach in itself will not guarantee that the folate content of the administered foods is equivalent to the FA dose being administered in parallel. In order to ensure equivalence all meals and snacks for the intervention period (whether or not it is conducted in a metabolic unit) would need to be prepared and analysed before they are consumed. The alternative, verifying the folate content of the intervention diet after its administration (by duplicate meal analysis, for example), has proven to be problematic even in highly-controlled studies such as that of Brouwer *et al.* (1999), because analysis of administered foods often reveals folate levels not equivalent to the FA dose, necessitating the use of a correction factor at the analysis stage. The major reason why the amount of folates in a meal is often found on analysis to differ from that targetted is primarily a result of the well-documented underestimation of the folate content of foods

in current Food Tables. Using newer food folate methodology based on trienzyme (protease, amylase and conjugase) treatment (Tamura *et al.* 1997), much higher values have been reported recently for folates in various plant and animal foods and in composite meals (McKillop *et al.* 2002; Pentieva *et al.* 2002) compared with published folate values generated using traditional folate methodology. This finding, if confirmed, may have major implications for the calculation of dietary folate intake (and therefore folate recommendations) and requires further investigation.

Conclusions

Food folate bioavailability and the factors that affect it are poorly understood. These considerations are important, given the established and emerging roles for folate in disease prevention on the one hand, and the widespread under-provision of folate in typical diets in most European countries on the other. A number of methodological approaches have been used to address food folate bioavailability, but there are some major limitations that may confound the interpretation of studies using these various approaches. Chronic studies with food folates are likely to give the most meaningful results in future research, but only if they are based on robust and tightly-controlled protocols to ensure the delivery of food folate and FA at equivalent doses throughout the intervention period. This challenge is a considerable one if folate bioavailability in whole diets is to be accurately assessed in order to provide quantifiable data on which to base food policy. In addition to the incomplete bioavailability of food folates generally evident from the literature, the poor stability of food folates (particularly in green vegetables) during cooking is an additional factor contributing to the limited ability of natural food folates to optimise folate status.

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